



King's Research Portal

DOI:

[10.1002/ejp.979](https://doi.org/10.1002/ejp.979)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Bannister, K., Lockwood, S., Goncalves, L., Patel, R., & Dickenson, A. H. (2017). An investigation into the inhibitory function of serotonin in diffuse noxious inhibitory controls in the neuropathic rat. *European Journal of Pain (United Kingdom)*, 21(4), 750-760. DOI: 10.1002/ejp.979

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

An investigation into the inhibitory function of serotonin in diffuse noxious inhibitory controls in the neuropathic rat

Running head: The inhibitory action of spinal serotonin in neuropathy

Kirsty Bannister^{1,2}, Stevie Lockwood¹, Leonor Goncalves¹, Ryan Patel¹ and Anthony H. Dickenson¹.

¹Department of Neuroscience, Physiology and Pharmacology, University College London, London, UK.

²Corresponding author. Address: Department of Neuroscience, Physiology and Pharmacology, University College London, Gower Street, London, WC1E 6BT. UK. Tel.: +44 2076793737; Fax: +44 2076797298. kirsty.bannister@ucl.ac.uk

Category for submission: Original article

This work was funded by a strategic award to the Wellcome Trust Pain Consortium (the London Pain Consortium) (KB, AHD, RP), European Commission FP7 (#HEALTH-F2-2013-602891) (LG) and UCL Medical Research Council quota award studentship (SL).

The authors declare that they have no conflicts of interest.

What does this study add? : Spinal application of selective serotonin reuptake inhibitors (SSRIs) reinstates diffuse noxious inhibitory controls (DNIC) acting on spinal neurons in neuropathic rats. Novel inhibitory actions via 5-HT₇ receptors now predominate, and effects

observed rely on an underlying inhibitory tonic noradrenergic component.

1. Introduction

Brainstem influences modulate spinal pain processing via descending pathways, whereby noradrenergic and serotonergic neurotransmission exerts bi-directional controls on pain perception (Bannister and Dickenson, 2016). Descending noradrenergic projections terminating in the dorsal horn of the spinal cord derive in particular from the locus coeruleus (LC). Micro-stimulation of these areas is anti-nociceptive via activation of the $\alpha 2$ -adrenoceptor (AR) (Jones and Gebhart 1986). Electrical stimulation of the rostral ventromedial medulla (RVM) evokes the spinal release of 5-hydroxytryptamine (5-HT), whose influence on spinal cord pain processing is contrasting (Eide and Hole 1993); activation of spinal 5-HT₃ receptors exacerbates pain signaling (Ali et al., 1996; Green et al., 2000; Guo et al., 2014; Suzuki et al., 2002) but activation of 5-HT₇ receptors can be anti-nociceptive (Brenchat et al., 2010; Dogrul et al., 2009; Dogrul and Seyrek 2006). Deciphering the role of the multiple 5-HT receptor subtypes in nociception remains complex (Kayser et al., 2011).

Spinal serotonergic and noradrenergic signaling is hypothesized a key underlying component of diffuse noxious inhibitory controls (DNIC) (Bannister et al., 2015; Chitour et al., 1982), a unique form of descending endogenous analgesia (De Broucker et al., 1990) in which the activity of trigeminal and wide dynamic range (WDR) neurons is constrained (Le Bars et al., 1979b). Originally proposed to derive from the subnucleus reticularis dorsalis (SRD) (Bouhassira et al., 1992), DNIC have a complex interplay between pathways comprising the dorsolateral funiculus (Okada-Ogawa et al., 2009). Human brain

imaging studies now show that signal changes associated with the human counterpart of DNIC include the SRD and parabrachial nucleus, with the former being controlled by cortical influences (Youssef et al., 2016a; b).

DNIC require a noxious conditioning stimulus (one pain inhibits another). In naïve rats, application of ear pinch activates DNIC, quantified as the inhibitory effect of the conditioning stimulus on WDR neuronal firing to hindpaw stimulation. The ear pinch was proposed to activate an α_2 AR noradrenergic control that overrode excitatory serotonergic events to trigger DNIC (Bannister et al., 2015). In spinal nerve ligated (SNL) animals there is an attenuation of α_2 AR-mediated inhibitions and an increase in 5-HT₃ receptor-mediated nociception (Rahman et al., 2008a; Suzuki et al., 2004) (Dogrul et al., 2009) and DNIC were shown completely abolished. An important role for descending serotonergic inhibitory pathways in DNIC was postulated (Chitour et al., 1982). Application of the 5-HT₃ receptor antagonist ondansetron revealed DNIC in SNL animals, indicative of an underlying, now-dominant, 5-HT₃ receptor-mediated facilitation restoring the normal balance in descending controls (Bannister et al., 2015).

Following neuropathy, in addition to sustained 5-HT facilitatory influences on the spinal cord, the density of 5-HT₇ receptors is increased in the dorsal horn (Brenchat et al., 2010). Given the complexity of serotonergic mechanisms in these spinal events, here we use selective serotonin reuptake inhibitors (SSRIs) to gauge the effect of an exaggerated spinal serotonergic content on the

expression of DNIC after neuropathy. We attempt to identify which 5-HT receptor actions predominate, nociceptive or anti-nociceptive, and whether any effects observed rely on an underlying inhibitory tonic noradrenergic component.

2. Methods

2.1. Animals

Male Sprague-Dawley rats (250-300g, Biological Services, UCL, UK) were used for electrophysiological experiments. Animals were group housed on a 12h:12h light-dark cycle. Food and water were available ad libitum. All procedures described were approved by the Home Office and adhered to the Animals (Scientific Procedures) Act 1986. Every effort was made to reduce animal suffering and the number of animals used in accordance with the IASP ethic guidelines (Zimmermann 1983). Total number of naïve animals used in this study = 16. Total number of SNL animals used in this study = 44.

2.2. Spinal nerve ligation surgery

Spinal nerve ligation (SNL) surgery was performed as described previously (Kim and Chung 1992). Rats (120-140g) were maintained under 2% v/v isoflurane anaesthesia delivered in a 3:2 ratio of nitrous oxide and oxygen. Under aseptic conditions, a paraspinal incision was made and the left tail muscle excised. Part of the L5 transverse process was removed to expose the L5 and L6 spinal nerves, which were then isolated with a glass nerve hook (Ski-Ry Ltd, London, UK) and ligated with a non-absorbable 6-0 braided silk thread proximal to the formation of the sciatic nerve. The surrounding skin and muscle were closed with absorbable 3-0

sutures. All rats were monitored for normal behaviours (grooming and mobility) and weight gain post-surgery.

2.3. Electrophysiology

In vivo electrophysiology experiments were conducted on post-operative days 14-18 (SNL-operated animals) or weight/age-matched naive rats as previously described (Urch and Dickenson 2003). Briefly, animals were anaesthetised and maintained for the duration of the experiment with isoflurane (1.5%) delivered in a gaseous mix of N₂O (66%) and O₂ (33%). A laminectomy was performed to expose the L4-5 segments of the spinal cord. Extracellular recordings were made from deep dorsal horn neurons (lamina V-VI) using parylene coated tungsten electrodes (A-M systems, USA). All the neurons recorded were wide dynamic range (WDR) and responded to natural stimuli including brush, low and high intensity mechanical and thermal stimuli in a graded manner with coding of increasing intensity.

The peripheral receptive field (hind paw) was stimulated using punctate mechanical stimuli (von Frey filaments 8, 26 and 60 g) and the number of action potentials fired in 5 s was recorded. Data were captured and analysed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design; rate functions).

Three baseline responses to mechanical stimuli as detailed above were characterised for each neuron before DNIC and subsequent pharmacological assessment (a drug study was carried out on one neuron per animal only).

2.4. DNIC study design

Extracellular recordings were made from one WDR neuron per animal by stimulating the hind paw peripheral receptive field and then repeating in the presence of ear pinch. Ear pinch was chosen as the conditioning stimulus since this part of the body is distant from the sciatic territory where the neuropathy was performed. The number of action potentials fired in 5 s was recorded for each test. Baseline responses were calculated from the mean of 2 trials. Each trial consisted of 3 consecutive stable responses to 8, 26 and 60g von Frey filaments applied to the hind paw (where all neurons met the inclusion criteria of <10% variation in action potential firing for all mechanically-evoked neuronal responses). This was then followed by consecutive responses to the same mechanical stimuli (8, 26 and 60g von Frey filaments) in the presence of DNIC. Precisely, DNIC was induced using a noxious ear pinch (15.75 x 2.3 mm Bulldog Serrefine, Interfocus, Linton, UK) on the ear ipsilateral to the neuronal recording whilst concurrent to this the peripheral receptive field was stimulated using the von Frey filaments listed. DNIC was quantified as an inhibitory effect on neuronal firing during ear pinch. A one-minute non-stimulation recovery period was allowed between each test in the trial. Following this, for pre-drug neuronal recordings, a 10-minute non-stimulation recovery period was allowed before the entire process was repeated and data for control trial number 2 was collected.

2.5. Drug administration

Following collection of pre-drug baseline control data as outlined in section 2.4, the drugs listed below were administered (one neuron

per animal). Each individual drug dose effect was followed for up to 60 minutes with tests carried out at 2 time points (20 and 40 minutes). For each time point, a trial consisted of 1) 3 consecutive stable responses to 8, 26 and 60g von Frey filaments (where all neurons met the inclusion criteria of <10% variation in action potential firing for all mechanically-evoked neuronal responses) followed by 2) consecutive responses to 8, 26 and 60g von Frey filaments with concurrent ear pinch. For post-drug DNIC effects, maximal changes from pre-drug DNIC responses are presented in the graphs for figures 1 -4.

The following drugs were used: Selective serotonin reuptake inhibitors (SSRI's) citalopram or fluoxetine (100µg) (Fuller et al., 1974; Hyttel 1977; Messing et al., 1975) (Tocris UK and Sigma UK respectively) were dissolved in saline (50µl) and delivered alone in a volume of 50µl or, in some instances, delivered with α_2 adrenoceptor antagonist atipamezole (100µg) (Rahman et al., 2008a) (Sigma UK) in a solution of 97% normal saline, 2% cremophor and 1% DMSO (50µl); 5-HT₇ receptor antagonist SB269970 (100µg) (Hagan et al., 2000) (Tocris UK) was dissolved in saline (50µl) and delivered to the spinal cord alone or, in some instances, in combination with fluoxetine or citalopram (100µg) in the same solution. For systemic administration of SSRI (10mg/kg for citalopram and 20mg/kg for fluoxetine), citalopram or fluoxetine were administered via subcutaneous injection.

2.6. Statistical analysis

Statistical analyses were performed using SPSSV22 (IBM, Armonk, NY). All data plotted in figures 1-4 are the raw firing rates,

representing the mean \pm SEM. Statistical differences in the neuronal responses (raw firing rates) (dependent variable) observed following ear pinch (independent variable) were determined using a 2-way repeated measures (RM) ANOVA followed by a Bonferroni post-hoc test for paired comparisons. Asterisks denote statistically significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3. Results

3.1. DNIC are present in naïve rats and absent in spinal nerve ligated rats

Throughout this study DNIC were induced by a noxious ear pinch applied to the ear ipsilateral to the neuron being recorded. The presence of DNIC was confirmed by a concurrent reduction in deep dorsal horn wide dynamic range (WDR) neuronal firing to stimulation of the hind paw peripheral receptive field in naïve animals ($n = 10$). This was compared to the magnitude of DNIC in spinal nerve ligated (SNL) (examined 14 days post-SNL surgery, $n = 22$) animals. Because previously no difference in the level of neuronal inhibition upon activation of DNIC was observed between naïve and sham-operated animals (Bannister et al., 2015), naïve animals only were used in this study as the control group. Activation of DNIC by heterotopic application of an ear pinch (conditioning stimulus) significantly and dramatically reduced the WDR neuronal response to non-noxious (8g) and noxious mechanical (26 and 60g) stimuli in naïve animal groups (2-way RM ANOVA; $P < 0.001$, $F_{(1,9)} = 194.42$. 40, 29 and 30% inhibition to 8, 26 and 60g vF respectively, $P < 0.001$ for all forces; Bonferroni post-hoc) (Figure 1A, C). Remarkably, and as observed previously (Bannister et al., 2015), when the magnitude of

DNIC was examined in SNL rats no reduction in WDR neuronal response to mechanical stimuli was observed upon application of the conditioning stimulus (2-way RM ANOVA; $P > 0.05$, $F_{(1,21)} = 3.32$. 10 and 6% increase in firing rate and 0% inhibition to 8, 26 and 60g vF respectively. $P > 0.05$ for all forces Bonferonni post hoc) (Figure 1B, D), thereby demonstrating a complete lack of DNIC in these animals. The degree of inhibition was comparable (30-40%) for the three vF filament stimulation intensities employed in keeping with the original studies where the conditioning stimulus modulates both non noxious and noxious stimuli (Le Bars et al., 1979; Bannister et al., 2015).

3.2. DNIC are revealed in SNL rats following spinal application of the SSRI citalopram

Here we investigated the effect of increasing spinal 5-HT content on the expression of DNIC in SNL animals. Following spinal application of the selective serotonin reuptake inhibitor (SSRI) citalopram (100µg, n = 6) the degree of inhibition produced by application of a noxious conditioning stimulus was dramatic and now WDR neuronal responses to noxious mechanical stimuli were significantly reduced (2-way RM ANOVA; $P < 0.01$, $F_{(1,5)} = 29.26$. 22, 46 and 38% inhibition to 8, 26 and 60g vF respectively in SNL animals, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively; Bonferroni post hoc) (Figure 2A, C). These data demonstrate an inhibitory effect of the elevated spinal 5-HT that is sufficient for DNIC to be revealed in neuropathic animals.

3.3. The restoration of DNIC in SNL rats following spinal application of citalopram is prevented by the $\alpha 2$ AR inhibitor atipamezole or the 5-HT7 receptor antagonist SB269970

Spinal application of the SSRI citalopram restored DNIC in SNL animals (Figure 2A, C). We aimed to identify the receptor-mediated action responsible for this inhibitory effect. Remarkably, in the presence of SB269970, a 5-HT7 receptor antagonist (Hagan et al., 2000), spinal application of citalopram no longer reduced WDR neuronal responses to mechanical stimuli upon application of the conditioning stimulus ($n = 6$) (2-way RM ANOVA; $P > 0.05$, $F_{(1,5)} = 0.025$. 12 and 5% increase in firing rate and 0% inhibition to 8, 26 and 60g vF respectively, $P > 0.05$ for all forces; Bonferroni post hoc) (Figure 2C, F), thereby demonstrating a complete blockade of DNIC and suggestive of an inhibitory action of excess spinal 5-HT via activation of the 5-HT7 receptor.

There is pre-existing evidence for the involvement of noradrenaline function in generating DNIC (Bannister et al., 2015; Peters et al., 2015). In order to extend these findings we examined the effect of dual spinal application of citalopram plus $\alpha 2$ AR antagonist atipamezole on the expression of DNIC in SNL animals. Intriguingly now we observed no reduction in WDR neuronal response to mechanical stimuli upon application of the conditioning stimulus ($n = 6$) (2-way RM ANOVA; $P > 0.05$, $F_{(1,5)} = 0.131$. 11% increase in firing rate, 3% inhibition and 2% increase in firing rate to 8, 26 and 60g vF respectively, $P > 0.05$ for all forces; Bonferroni post hoc) (Figure 2B, E). Once again a complete blockade of DNIC was demonstrated, and these data support the premise that an inhibitory noradrenergic tone must be present spinally for the expression of DNIC.

Of concern was a prior report relating to the possible antagonistic effect of SB269970 at the $\alpha 2$ AR (Foong and Bornstein 2009). Previous work has shown that spinal application of $\alpha 2$ AR antagonist atipamezole blocks the expression of DNIC in naïve rats, such that WDR neuronal responses are no longer inhibited during concurrent noxious ear pinch (Bannister et al., 2015). However in the present study, following topical application of SB266970 alone to the spinal cord of naïve rats, there was no change in baseline responses nor the efficacy of DNIC; WDR neuronal responses were still inhibited by concurrent noxious ear pinch ($n = 6$) (2-way RM ANOVA; $P > 0.01$, $F_{(1,5)} = 20.99$. 27, 20 and 35% inhibition to 8, 8, 26 and 60g vF respectively, $P > 0.05$ and $P > 0.01$ respectively; Bonferroni post hoc) (Figure 3A). This suggests that, at this concentration and for this protocol at least, SB269970 is not antagonizing the $\alpha 2$ AR.

Note: Spinal application of vehicle control (97% normal saline, 2% cremophor, 1% DMSO) had no effect on baseline neuronal firing rates or on the level of WDR neuronal inhibition observed upon concurrent ear pinch (data not shown).

3.4. DNIC are revealed in SNL animals following spinal application of SSRI fluoxetine through 5-HT7 and $\alpha 2$ AR mechanisms

In order to verify the revelatory effect of the evidently most highly selective SSRI citalopram (Pawlowski et al., 1985) on DNIC we repeated the experiments above using SSRI fluoxetine (Fuller et al., 1991). Similarly to what we observed with spinal application of citalopram, in the presence of spinal fluoxetine (100 μ g, $n = 6$) there was a significant and dramatic reduction of WDR neuronal responses to noxious mechanical stimuli upon simultaneous ear pinch, which

achieved levels of inhibition practically identical to those observed following spinal application of citalopram (2-way RM ANOVA; $P < 0.001$, $F_{(1,5)} = 40.42$, 38 and 34% inhibition to 8, 26 and 60g vF respectively in SNL animals, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively; Bonferroni post hoc) (Figure 3B). Once again, dual spinal application of fluoxetine and 5-HT₇ receptor antagonist SB269970 or fluoxetine and α_2 AR antagonist atipamezole resulted in no reduction of WDR neuronal response to mechanical stimuli upon application of the conditioning stimulus ($n = 5$ and $n = 6$ respectively) (2-way RM ANOVA; $P > 0.05$, $F_{(1,4)} = 0.024$, 7% increase in firing rate and 2 and 2% inhibition to 8, 26 and 60g vF respectively, $P > 0.05$ for all forces; Bonferroni post hoc; $P > 0.05$ for all forces; 2-way RM ANOVA; $P > 0.05$, $F_{(1,5)} = 1.33$, 7, 2 and 7% inhibition to 8, 26 and 60g vF respectively, Bonferroni post hoc) (Figure 3C and D). Once more, these data demonstrate an inhibitory effect of the elevated spinal 5-HT that is sufficient for DNIC to be revealed in neuropathic animals. These results validate the effects observed in the presence of spinal citalopram.

3.5. DNIC are not present in SNL rats following systemic application of citalopram or fluoxetine

We investigated the expression of DNIC in SNL animals following systemic administration of citalopram ($n = 4$, 10mg/kg) or fluoxetine ($n = 5$, 20mg/kg). Contrasting those results observed with spinal application of drug, systemic administration of citalopram or fluoxetine resulted in no reduction of WDR neuronal response to mechanical stimuli upon application of the conditioning stimulus (2-way RM ANOVA; $P > 0.05$, $F_{(1,3)} = 0.00022$ for citalopram, 4% increase

in firing rate, 0 and 4% inhibition to 8, 26 and 60g vF respectively, $P > 0.05$ for all forces; Bonferroni post hoc; 2-way RM ANOVA; $P > 0.05$, $F_{(1,4)} = 0.136$ for fluoxetine. 18% increase in firing rate, 6 and 4% inhibition to 8, 26 and 60g vF respectively, $P > 0.05$ for all forces; Bonferroni post hoc) (Figure 4A and 4B).

Discussion

Diffuse noxious inhibitory controls (DNIC) are a powerful manifestation of endogenous analgesia that describes the phenomena whereby application of strong pain to one part of the body inhibits pain in multiple remote body regions. As observed here and previously (Bannister et al., 2015; Le Bars et al., 1979a) the inhibitory action of this part-opioid descending pain modulatory pathway on trigeminal and wide dynamic range (WDR) neurons upon presentation of a conditioning stimulus is robust and reliable. Conditioned pain modulation (CPM), the human counterpart of DNIC, utilizes a clinical paradigm involving a distant painful conditioning stimulus used to affect a test stimulus (Yarnitsky 2010). Like DNIC CPM is absent in tetraplegics (Roby-Brami et al., 1987) and both are reduced following neuropathy to varying degrees (Bannister et al., 2015; Niesters et al., 2014; Yarnitsky 2010). DNIC clearly involve noradrenergic inhibitory pathways; they are abolished by blockade of the α_2 AR (Bannister et al., 2015; Peters et al., 2015) and after spinal nerve ligation (SNL), but restored by enhancing synaptic levels of NA. This is the case with failed CPM in patients with neuropathy, now a predictor of the efficacy of duloxetine, a serotonin-noradrenaline reuptake inhibitor (SNRI) (Yarnitsky et al., 2012).

Further, restoration of CPM was observed following treatment with tapentadol, a mu opioid receptor agonist and noradrenaline reuptake inhibitor (MOR-NRI) (Niesters et al., 2014). This corroborates the animal data that noradrenaline-mediated actions at the $\alpha 2$ AR and opposing facilitations via 5-HT-mediated actions at 5-HT₃ receptors are accountable, in part, for this change. The role of 5HT in pain modulation is complex due to the multiplicity of receptors. DNIC require inhibitory actions of 5-HT (Chitour et al., 1982) but after SNL are prevented from modulating spinal neuronal activity by enhanced 5-HT₃ receptor facilitations (Bannister et al., 2015). Here, the role of 5-HT in DNIC is investigated further.

We have reaffirmed unequivocally that DNIC are absent in SNL animals. Now we demonstrate reinstatement of DNIC in neuropathic animals following spinal, not systemic, application of selective serotonin reuptake inhibitors (SSRI's) citalopram or fluoxetine, which presumably act to increase spinal 5-HT content. We provide a pharmacological basis for this revelatory effect since it was abolished completely upon joint spinal application of either SSRI with the 5-HT₇ receptor antagonist SB269970 and thus support pre-existing evidence of an anti-nociceptive role of 5-HT₇ receptors; in chronic pain models descending pain inhibitory pathways from the RVM provoked by morphine were previously shown to rely on activation of spinal 5-HT₇ receptors (Dogrul et al., 2009). Meanwhile the anti-depressant tianeptine had an anti-allodynic effect in a rat model of neuropathy mediated via 5-HT₇ receptors located on spinal GABAergic interneurons (Lin et al., 2015). Additionally, in a mouse model of neuropathy, activation of 5-HT₇ receptors was shown to

reduce mechanical and thermal hypersensitivities, and a significant increase in 5-HT₇ receptor densities in the dorsal horn of the spinal cord was shown (Brenchat et al., 2010). In contrast a pro-nociceptive role for the 5-HT₇ receptors was previously reported in the rat formalin test (Rocha-Gonzalez et al., 2005). However overwhelming is data supporting a role of the 5-HT₇ receptors in modulation of neuropathic pain.

It is likely that the levels of 5-HT in the spinal cord and elsewhere determine the direction of effect of the transmitter and the particular receptor. Endogenous 5-HT is facilitatory (Rahman et al., 2006) and there is a resting 5-HT₃ receptor facilitation that is enhanced after neuropathy and in other pain states (Bannister and Dickenson 2016; Dogrul and Seyrek 2006; Suzuki et al., 2002) probably due to increased levels of 5-HT. Yet when spinal 5-HT is elevated further by the SSRIs, DNIC now utilises a 5-HT₇ receptor inhibitory system. SB269970 had no effect alone when given in the absence of elevated 5-HT. Interestingly, the 5-HT₇ receptor mediates the spinal analgesia produced by morphine acting through descending controls (Dogrul and Seyrek 2006). The facilitatory role of the 5-HT₃ receptor may be overcome when levels of 5-HT are very high. The lack of effect of the SSRIs given systemically and their modest effects in patients compared to SNRIs likely results from their inability to raise spinal 5-HT (or indeed NA) sufficiently. Recently citalopram reduced both brain activation and pain ratings in volunteers but importantly the effects of the drug were dependent on serotonin transporter (5-HTT) gene polymorphisms (Ma et al., 2016). Intriguingly the degree of CPM

in volunteers is compromised in the low 5-HTT expressing group (Lindstedt et al., 2011).

Initially perplexing was the observation that the DNIC-revealing effect of spinal application of citalopram or fluoxetine was completely abolished by the α_2 AR antagonist atipamezole. However pre-existing evidence of the involvement of noradrenaline function in generating DNIC remain (Bannister et al., 2015; Peters et al., 2015). We extend this theory by hypothesising that, even in the presence of a reduced noradrenergic inhibitory control via the α_2 AR (as is understood to be the case in neuropathy), this component of descending modulation is nonetheless vital for the expression of DNIC, even in the presence of an enhanced now-inhibitory action of serotonin via activation of the 5-HT₇ receptors. It remains unclear as to the mechanisms behind the spinal interactions between these 5-HT and NA pathways but there may be cross talk or reciprocal controls between the two monoamines and the terminals of their respective descending pathways. Another conceivable substrate for the NA and 5HT interactions could be the spinal neurons that drive these systems. A population of superficial NK1 receptor expressing neurons is required for activation of RVM neurons, DNIC and both the descending α_2 AR mediated-inhibitions and 5-HT₃ facilitations (Rahman et al., 2008b; Suzuki et al., 2002). Any alteration in activity of these neurons produced by elevated spinal 5HT could alter the balance between these descending controls, exactly those we are studying here.

The SSRI's are largely ineffective as a treatment for neuropathic pain (Finnerup and Attal 2015). Correspondingly when given systemically in SNL animals we observed no suggestive activation of DNIC. However, a local spinal application of citalopram or fluoxetine did restore DNIC. The selectivity of particular SSRI's is a moot point, not least because of the reported effects on noradrenergic function. While fluoxetine is considered a useful tool for studying 5-HT neurotransmission (Fuller et al., 1991; Wong et al., 1985), questions regarding its selectivity have been raised following a reported effect on noradrenergic function (O'Flynn et al., 1991). Citalopram is arguably the most selective SSRI agent (Hyttel 1982; Pawlowski et al., 1985). Here, the question of whether or not there is a noradrenergic component to the actions of either fluoxetine or citalopram is crucially relevant. Conceivably the drugs may have noradrenaline actions in vivo. In terms of DNIC activation in SNL animals we can be confident that there is a 5-HT₇ receptor-mediated inhibition, but an adrenergic component to DNIC must exist given that blocking the α_2 AR with antagonist atipamezole in naïve animals abolishes the inhibitory effect on WDR neuronal responses. In SNL animals it could be hypothesised that there is a tonic, albeit much lower, spinal noradrenergic drive present that exerts inhibitory actions via the α_2 AR insufficient to allow DNIC. But now, in the presence of increased spinal 5-HT content as is the case following spinal application of SSRI (which conceivably could desensitize 5-HT₃ mediated facilitations), this reduced noradrenergic drive is now able to generate the inhibitory actions of DNIC in the presence of the novel actions of 5-HT at the inhibitory 5-HT₇ receptor.

Is the lack of effect of systemic SSRIs in terms of restoration of DNIC in SNL animals due to a neural 5-HT-NA interaction? Noradrenergic inputs to the RVM are known to effect nociceptive modulatory neurons by targeting both On and Off cells (Meng et al., 1997) and a role for excitatory $\alpha 1$ AR's and inhibitory $\alpha 2$ AR's in the nucleus raphe magnus in opioid analgesia has been proposed (Bie et al., 2003). Stimulation of the RVM evokes the spinal release of 5-HT. Likely there is cross-talk between 5-HT and NA within the midbrain and brainstem as well as at spinal levels. The periaqueductal grey (PAG) inhibits nociceptive inputs to the dorsal horn of the spinal cord through $\alpha 2$ AR (Budai et al., 1998). With systemic dosing these proposed supra-spinal interactions between the monoamines may be the basis for changes in descending systems that now do not culminate in inhibition of spinal nociception. Roles of the 5-HT₁ receptor as well as interactions with GABA and other transmitters may well also be relevant.

Overall, the data reveal complex bidirectional pharmacological substrates for descending controls with changes in their balances after nerve injury and after drug treatments. The translational value of DNIC to CPM that can be gauged in the clinic suggests that monoamine modulation has promise for treating pain in patients, with noradrenergic mechanisms being essential whereas the effects of serotonin appear to depend on individual levels. The present study supports these approaches in patients with neuropathy, but since DNIC and CPM are diffuse whole body inhibitory controls these concepts could be extended to other patients such as those with fibromyalgia.

Acknowledgements

All persons contributing to this piece of work are listed as authors.

Author Contributions

KB contributed to conception and design of study, acquisition of data, analysis and interpretation of data, SNL surgeries and writing the manuscript, SL contributed to design of study and acquisition of data, LG performed SNL surgeries, RP contributed to data acquisition, AHD contributed to conception and design of study and finalization of manuscript. All authors discussed the results and commented on the manuscript.

References

- Ali Z, Wu G, Kozlov A, Barasi S. The role of 5HT₃ in nociceptive processing in the rat spinal cord: results from behavioural and electrophysiological studies. *Neurosci Lett* 1996;208: 203-207.
- Bannister K and Dickenson AH. What do monoamines do in pain modulation? *Curr Opin Support Palliat Care* 2016;10: 143-148.
- Bannister K, Patel R, Goncalves L, Townson L, Dickenson AH. Diffuse noxious inhibitory controls and nerve injury: restoring an imbalance between descending monoamine inhibitions and facilitations. *Pain* 2015;156: 1803-1811.
- Bie B, Fields HL, Williams JT, Pan ZZ. Roles of alpha₁- and alpha₂-adrenoceptors in the nucleus raphe magnus in opioid analgesia and opioid abstinence-induced hyperalgesia. *J Neurosci* 2003;23: 7950-7957.
- Bouhassira D, Bing Z, Le Bars D. Effects of lesions of locus coeruleus/subcoeruleus on diffuse noxious inhibitory controls in the rat. *Brain Res* 1992;571: 140-144.
- Brenchat A, Nadal X, Romero L, Ovalle S, Muro A, Sanchez-Arroyos R, Portillo-Salido E, Pujol M, Montero A, Codony X, Burgueno J, Zamanillo D, Hamon M, Maldonado R, Vela JM. Pharmacological activation of 5-HT₇ receptors reduces nerve injury-induced mechanical and thermal hypersensitivity. *Pain* 2010;149: 483-494.
- Budai D, Harasawa I, Fields HL. Midbrain periaqueductal gray (PAG) inhibits nociceptive inputs to sacral dorsal horn nociceptive neurons through alpha₂-adrenergic receptors. *J Neurophysiol* 1998;80: 2244-2254.
- Chitour D, Dickenson AH, Le Bars D. Pharmacological evidence for the involvement of serotonergic mechanisms in diffuse noxious inhibitory controls (DNIC). *Brain Res* 1982;236: 329-337.
- De Broucker T, Cesaro P, Willer JC, Le Bars D. Diffuse noxious inhibitory controls in man. Involvement of the spinoreticular tract. *Brain* 1990;113 (Pt 4): 1223-1234.
- Dogrul A, Ossipov MH, Porreca F. Differential mediation of descending pain facilitation and inhibition by spinal 5HT-3 and 5HT-7 receptors. *Brain Res* 2009;1280: 52-59.
- Dogrul A and Seyrek M. Systemic morphine produce antinociception mediated by spinal 5-HT₇, but not 5-HT_{1A} and 5-HT₂ receptors in the spinal cord. *Br J Pharmacol* 2006;149: 498-505.
- Eide PK and Hole K. The role of 5-hydroxytryptamine (5-HT) receptor subtypes and plasticity in the 5-HT systems in the regulation of nociceptive sensitivity. *Cephalalgia* 1993;13: 75-85.
- Finnerup NB and Attal N. Pharmacotherapy of neuropathic pain: time to rewrite the rulebook? *Pain management* 2015.
- Foong JP and Bornstein JC. 5-HT antagonists NAN-190 and SB 269970 block alpha₂-adrenoceptors in the guinea pig. *Neuroreport* 2009;20: 325-330.
- Fuller RW, Perry KW, Molloy BB. Effect of an uptake inhibitor on serotonin metabolism in rat brain: studies with 3-(p-trifluoromethylphenoxy)-N-methyl-3-phenylpropylamine (Lilly 110140). *Life sciences* 1974;15: 1161-1171.

- Fuller RW, Wong DT, Robertson DW. Fluoxetine, a selective inhibitor of serotonin uptake. *Med Res Rev* 1991;11: 17-34.
- Green GM, Scarth J, Dickenson A. An excitatory role for 5-HT in spinal inflammatory nociceptive transmission; state-dependent actions via dorsal horn 5-HT(3) receptors in the anaesthetized rat. *Pain* 2000;89: 81-88.
- Guo W, Miyoshi K, Dubner R, Gu M, Li M, Liu J, Yang J, Zou S, Ren K, Noguchi K, Wei F. Spinal 5-HT₃ receptors mediate descending facilitation and contribute to behavioral hypersensitivity via a reciprocal neuron-glial signaling cascade. *Mol Pain* 2014;10: 35.
- Hagan JJ, Price GW, Jeffrey P, Deeks NJ, Stean T, Piper D, Smith MI, Upton N, Medhurst AD, Middlemiss DN, Riley GJ, Lovell PJ, Bromidge SM, Thomas DR. Characterization of SB-269970-A, a selective 5-HT(7) receptor antagonist. *Br J Pharmacol* 2000;130: 539-548.
- Hyttel J. Neurochemical characterization of a new potent and selective serotonin uptake inhibitor: Lu 10-171. *Psychopharmacology (Berl)* 1977;51: 225-233.
- Hyttel J. Citalopram--pharmacological profile of a specific serotonin uptake inhibitor with antidepressant activity. *Prog Neuropsychopharmacol Biol Psychiatry* 1982;6: 277-295.
- Jones SL and Gebhart GF. Characterization of coeruleospinal inhibition of the nociceptive tail-flick reflex in the rat: mediation by spinal alpha 2-adrenoceptors. *Brain Res* 1986;364: 315-330.
- Kayser V, Latremoliere A, Hamon M, Bourgoin S. N-methyl-D-aspartate receptor-mediated modulations of the anti-allodynic effects of 5-HT_{1B/1D} receptor stimulation in a rat model of trigeminal neuropathic pain. *Eur J Pain* 2011;15: 451-458.
- Kim SH and Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 1992;50: 355-363.
- Le Bars D, Dickenson AH, Besson JM. Diffuse noxious inhibitory controls (DNIC). I. Effects on dorsal horn convergent neurones in the rat. *Pain* 1979a;6: 283-304.
- Le Bars D, Dickenson AH, Besson JM. Diffuse noxious inhibitory controls (DNIC). II. Lack of effect on non-convergent neurones, supraspinal involvement and theoretical implications. *Pain* 1979b;6: 305-327.
- Lin H, Heo BH, Kim WM, Kim YC, Yoon MH. Antiallodynic effect of tianeptine via modulation of the 5-HT₇ receptor of GABAergic interneurons in the spinal cord of neuropathic rats. *Neurosci Lett* 2015;598: 91-95.
- Lindstedt F, Berrebi J, Greayer E, Lonsdorf TB, Schalling M, Ingvar M, Kosek E. Conditioned pain modulation is associated with common polymorphisms in the serotonin transporter gene. *PloS one* 2011;6: e18252.
- Ma Y, Wang C, Luo S, Li B, Wager TD, Zhang W, Rao Y, Han S. Serotonin transporter polymorphism alters citalopram effects on human pain responses to physical pain. *Neuroimage* 2016;135: 186-196.
- Meng XW, Budra B, Skinner K, Ohara PT, Fields HL. Noradrenergic input to nociceptive modulatory neurons in the rat rostral ventromedial medulla. *The Journal of comparative neurology* 1997;377: 381-391.

- Messing RB, Phebus L, Fisher LA, Lytle LD. Analgesic effect of fluoxetine hydrochloride (Lilly 110140), a specific inhibitor of serotonin uptake. *Psychopharmacol Commun* 1975;1: 511-521.
- Niesters M, Proto PL, Aarts L, Sarton EY, Drewes AM, Dahan A. Tapentadol potentiates descending pain inhibition in chronic pain patients with diabetic polyneuropathy. *Br J Anaesth* 2014;113: 148-156.
- O'Flynn K, O'Keane V, Lucey JV, Dinan TG. Effect of fluoxetine on noradrenergic mediated growth hormone release: a double blind, placebo-controlled study. *Biol Psychiatry* 1991;30: 377-382.
- Okada-Ogawa A, Porreca F, Meng ID. Sustained morphine-induced sensitization and loss of diffuse noxious inhibitory controls in dura-sensitive medullary dorsal horn neurons. *J Neurosci* 2009;29: 15828-15835.
- Pawlowski L, Nowak G, Gorka Z, Mazela H. Ro 11-2465 (cyan-imipramine), citalopram and their N-desmethyl metabolites: effects on the uptake of 5-hydroxytryptamine and noradrenaline in vivo and related pharmacological activities. *Psychopharmacology (Berl)* 1985;86: 156-163.
- Peters CM, Hayashida K, Suto T, Houle TT, Aschenbrenner CA, Martin TJ, Eisenach JC. Individual differences in acute pain-induced endogenous analgesia predict time to resolution of postoperative pain in the rat. *Anesthesiology* 2015;122: 895-907.
- Rahman W, D'Mello R, Dickenson AH. Peripheral nerve injury-induced changes in spinal $\alpha(2)$ -adrenoceptor-mediated modulation of mechanically evoked dorsal horn neuronal responses. *J Pain* 2008a;9: 350-359.
- Rahman W, Suzuki R, Hunt SP, Dickenson AH. Selective ablation of dorsal horn NK1 expressing cells reveals a modulation of spinal $\alpha(2)$ -adrenergic inhibition of dorsal horn neurones. *Neuropharmacology* 2008b;54: 1208-1214.
- Rahman W, Suzuki R, Webber M, Hunt SP, Dickenson AH. Depletion of endogenous spinal 5-HT attenuates the behavioural hypersensitivity to mechanical and cooling stimuli induced by spinal nerve ligation. *Pain* 2006;123: 264-274.
- Roby-Brami A, Bussel B, Willer JC, Le Bars D. An electrophysiological investigation into the pain-relieving effects of heterotopic nociceptive stimuli. Probable involvement of a supraspinal loop. *Brain* 1987;110 (Pt 6): 1497-1508.
- Rocha-Gonzalez HI, Meneses A, Carlton SM, Granados-Soto V. Pronociceptive role of peripheral and spinal 5-HT₇ receptors in the formalin test. *Pain* 2005;117: 182-192.
- Suzuki R, Morcuende S, Webber M, Hunt SP, Dickenson AH. Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. *Nature neuroscience* 2002;5: 1319-1326.
- Suzuki R, Rahman W, Hunt SP, Dickenson AH. Descending facilitatory control of mechanically evoked responses is enhanced in deep dorsal horn neurones following peripheral nerve injury. *Brain research* 2004;1019: 68-76.
- Urch CE and Dickenson AH. In vivo single unit extracellular recordings from spinal cord neurones of rats. *Brain research Brain research protocols* 2003;12: 26-34.

- Wong DT, Reid LR, Bymaster FP, Threlkeld PG. Chronic effects of fluoxetine, a selective inhibitor of serotonin uptake, on neurotransmitter receptors. *J Neural Transm* 1985;64: 251-269.
- Yarnitsky D. Conditioned pain modulation (the diffuse noxious inhibitory control-like effect): its relevance for acute and chronic pain states. *Current opinion in anaesthesiology* 2010;23: 611-615.
- Yarnitsky D, Granot M, Nahman-Averbuch H, Khamaisi M, Granovsky Y. Conditioned pain modulation predicts duloxetine efficacy in painful diabetic neuropathy. *Pain* 2012;153: 1193-1198.
- Youssef AM, Macefield VG, Henderson LA. Cortical influences on brainstem circuitry responsible for conditioned pain modulation in humans. *Hum Brain Mapp* 2016a;37: 2630-2644.
- Youssef AM, Macefield VG, Henderson LA. Pain inhibits pain; human brainstem mechanisms. *Neuroimage* 2016b;124: 54-62.
- Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983;16: 109-110.

Figure Legends

Figure 1

The effect of DNIC activation on response profiles of deep dorsal horn WDR neurons in naïve ($n = 10$) and spinal nerve ligated (SNL) ($n = 22$) rats is shown. All data are presented as the mean \pm standard error of the mean. Here, the DNIC effect is expressed as the maximal evoked change in neuronal response. In all experimental groups the evoked responses to mechanical stimuli were recorded before and after activation of DNIC. A noxious ear pinch ipsilateral to the neuronal receptive field significantly reduced the excitability of spinal neurons to simultaneous peripherally applied non-noxious and noxious stimuli in naïve animals (A). A noxious ear pinch ipsilateral to the neuronal receptive field had no statistically significant effect on the excitability of spinal neurons to peripherally applied stimuli in SNL rats (B). The first representative trace shows 3 control responses to von Frey filaments and a response profile following the simultaneous application of a noxious ear pinch. There is a statistically significant comparative reduction in neuronal action potential firing following activation of DNIC in naïve animals (C). There is no statistically significant reduction in neuronal action potential firing following activation of DNIC in SNL rats on the ipsilateral side (D). Traces represent single unit recordings. Columns represent number of spikes per second. Significant differences from baseline response: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 2

The effect of 1) spinal citalopram (100ug) and 2) spinal citalopram plus atipamezole (100ug) and 3) spinal citalopram plus SB269970 (100ug) on the response profiles of deep dorsal horn WDR neurons in SNL animals ($n = 6$ for all groups) is shown. All data are presented as the mean \pm standard error of the mean. Here the DNIC effect is expressed as the maximal evoked change in neuronal response. In all experimental groups the evoked responses to mechanical stimuli were recorded before and after activation of DNIC. In the presence of spinal citalopram following a noxious ear pinch the excitability of spinal neurons to simultaneous peripherally applied mechanical stimuli was significantly reduced (A). However when citalopram was applied in the presence of spinal atipamezole or SB-269970, noxious ear pinch now had no statistically significant effect on the excitability of spinal neurons to simultaneous peripherally applied non-noxious and noxious stimuli (B and C). The three representative traces each show one example of a pre-drug control and then the response profile following the simultaneous application of a noxious ear pinch, and then one example of a post-drug control and the response profile following the simultaneous application of a noxious ear pinch (D, E and F). Traces represent single unit recordings. Columns represent number of spikes per second. Significant differences from baseline response: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 3

The effect of 1) spinal SB269970 (100ug) ($n = 6$) on the response profiles of deep dorsal horn WDR neurons in naive animals and 2) spinal fluoxetine (100ug) ($n = 6$), 3) spinal fluoxetine plus atipamezole (100ug) ($n = 6$), 4) spinal fluoxetine plus SB269970

(100ug) (n = 5) on the response profiles of deep dorsal horn WDR neurons in SNL animals, is shown. All data are presented as the mean \pm standard error of the mean. Here the DNIC effect is expressed as the maximal evoked change in neuronal response. In all experimental groups the evoked responses to mechanical stimuli were recorded before and after activation of DNIC. In naïve animals following a noxious ear pinch the excitability of spinal neurons to simultaneous peripherally applied mechanical stimuli was significantly reduced in the presence of spinal SB269970 (A). In the presence of spinal fluoxetine following a noxious ear pinch the excitability of spinal neurons to simultaneous peripherally applied mechanical stimuli was significantly reduced in SNL animals (B). However in SNL animals when fluoxetine was applied in the presence of spinal atipamezole or SB269970, noxious ear pinch now had no statistically significant effect on the excitability of spinal neurons to simultaneous peripherally applied non-noxious and noxious stimuli (C and D). Significant differences from baseline response: * $P < 0.05$, ** $P < 0.01$.

Figure 4

The effect of systemic citalopram (n = 5) and systemic fluoxetine (n = 6) (10mg/kg and 20mg/kg respectively) on the response profiles of deep dorsal horn WDR neurons in SNL animals is shown. All data are presented as the mean \pm standard error of the mean. Here the DNIC effect is expressed as the maximal evoked change in neuronal response. In all experimental groups the evoked responses to mechanical stimuli were recorded before and after activation of DNIC. In the presence of systemic citalopram or systemic fluoxetine there was no reduction in excitability of spinal neurons to

simultaneous peripherally applied mechanical stimuli in the presence of noxious ear pinch (A and B).

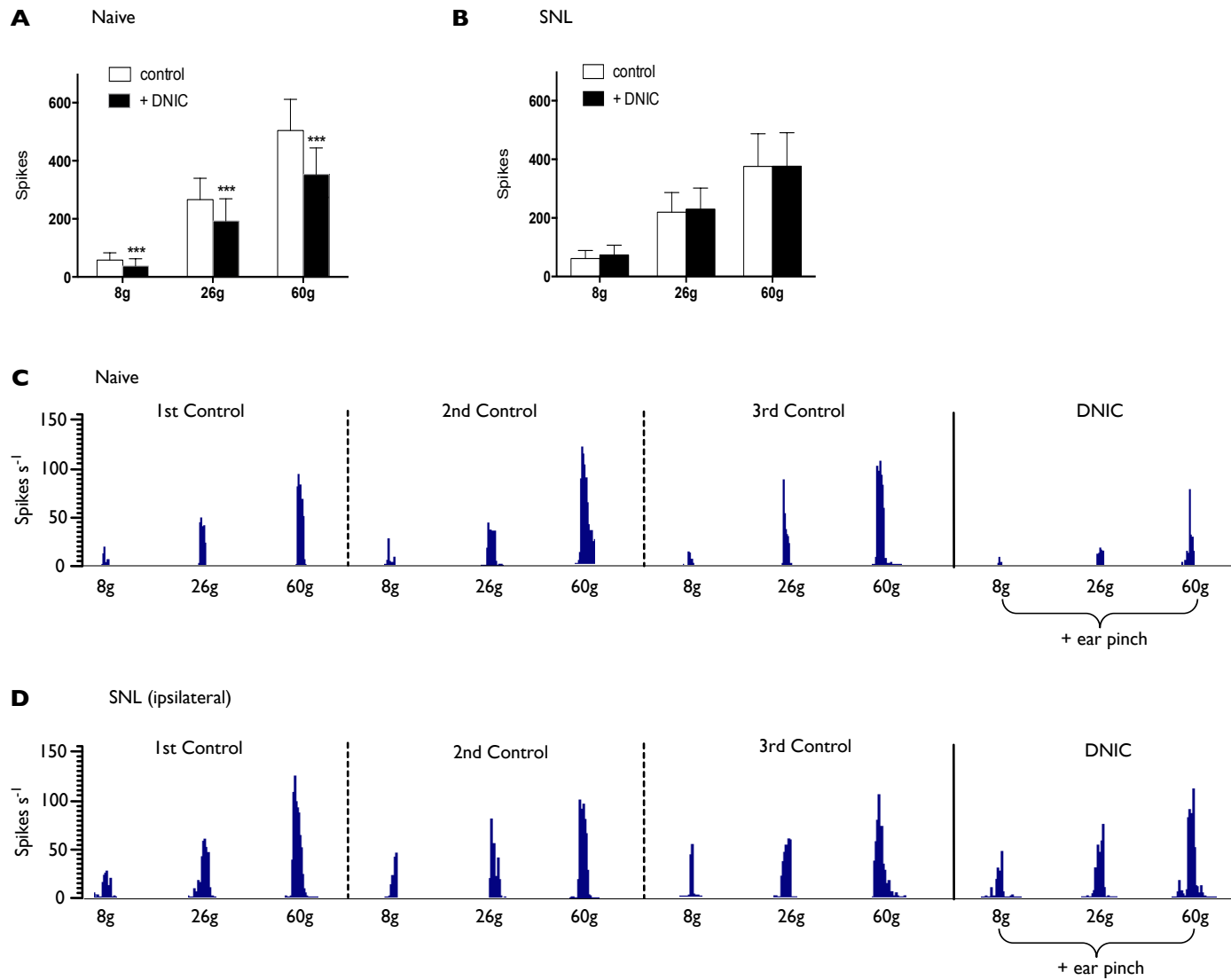
Figure I

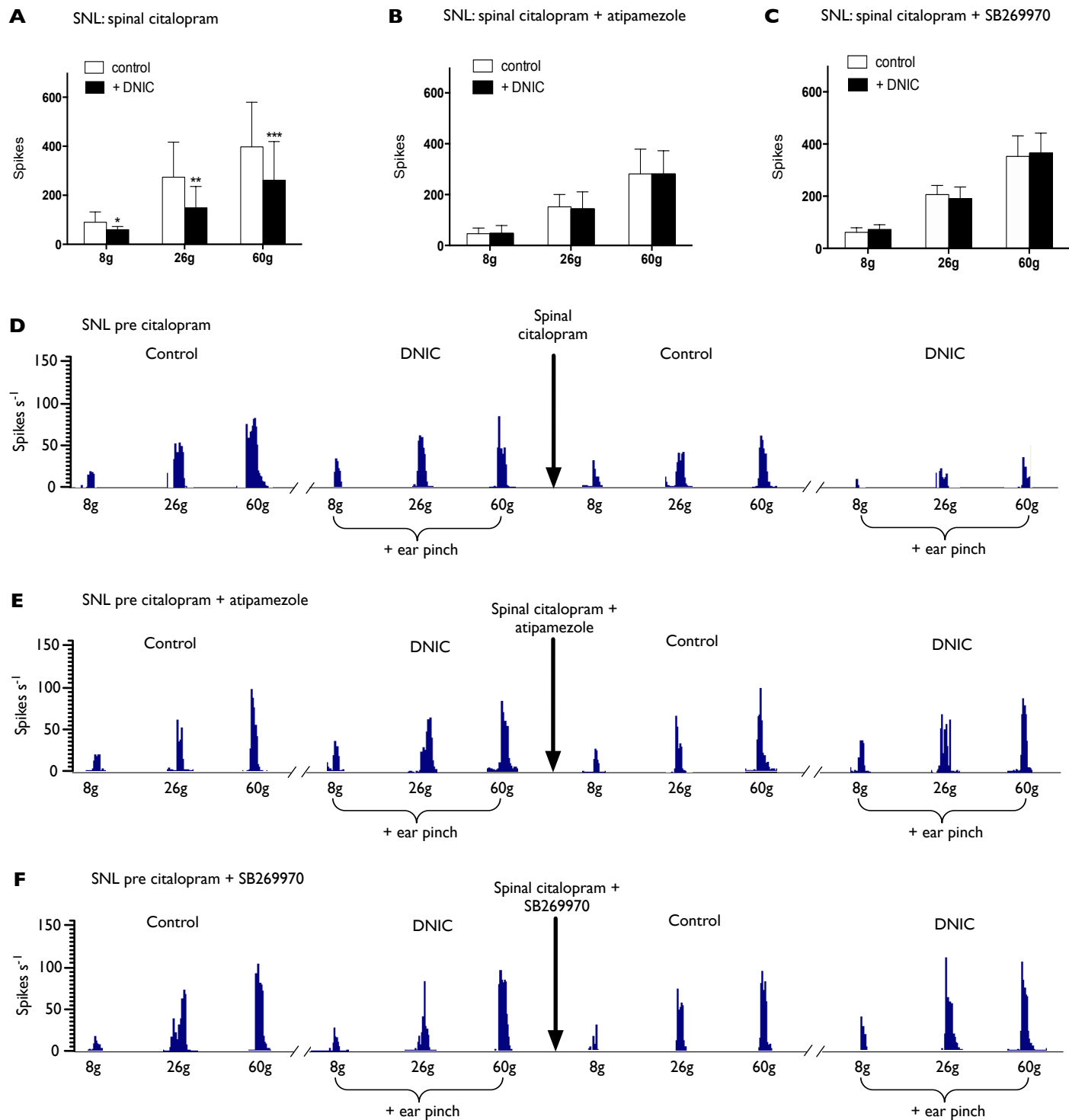
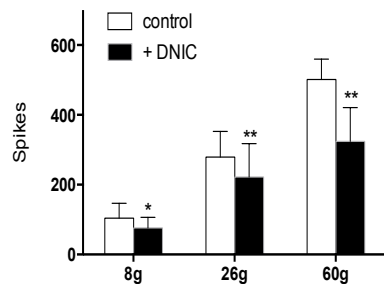
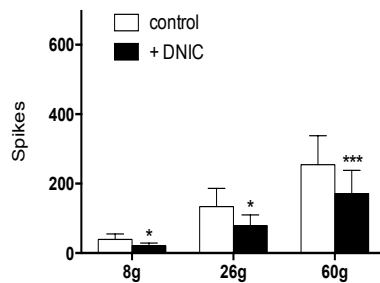
Figure 2

Figure 3

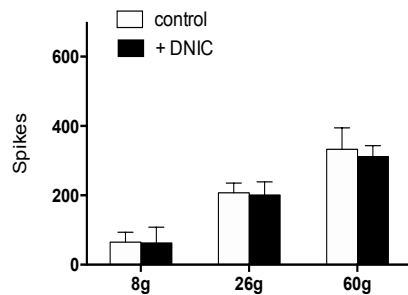
A Naive: spinal SB269970



B SNL: spinal fluoxetine



C SNL: spinal fluoxetine + atipamezole



D SNL: spinal fluoxetine + SB269970

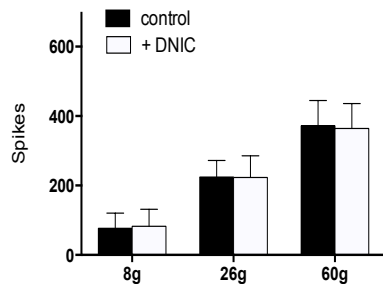
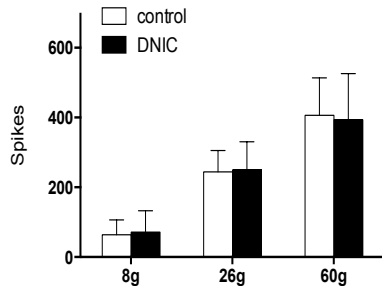


Figure 4

A SNL: systemic citalopram



B SNL: systemic fluoxetine

